Human parathyroid hormone does not influence human erythropoiesis in vitro

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Abstract

Background. Although renal anaemia is associated with secondary hyperparathyroidism, the relationship of both conditions remains obscure. Previously it was reported that high levels of bovine parathyroid hormone (PTH) did not inhibit in vitro human erythropoiesis, but whether human PTH inhibits in vitro human erythropoiesis has not been determined.

Method. To clarify the direct effects of human biologically active N-terminal (1–34) PTH and intact (1–84) PTH on human haematopoietic progenitor growth, we investigated colony assays of human erythropoiesis and granulomonopoiesis.

Results. Neither N-terminal PTH (300 ng/ml) nor intact PTH (5000 pg/ml) inhibited haematopoietic progenitor growth.

Conclusion. Our findings confirm that human PTH does not directly inhibit human erythropoiesis.

Key words: human parathyroid hormone; human haematopoietic progenitor growth

Introduction

Anaemia is a common complication in patients with chronic renal failure (CRF). Absolute and relative decrease in erythropoietin (Epo) production is the main contributor to anaemia [1,2]. Injection of recombinant human Epo is usually an effective treatment of renal anaemia, but some patients exhibit therapeutic failure [3–5]. As a cause of therapeutic failure, several inhibitors are considered to interfere with erythroid marrow function. Secondary hyperparathyroidism (2-HPT) is one factor [6]. In fact, several investigators described the improvement of anaemia in patients with CRF following subtotal parathyroidectomy [5,7–9]. However, the exact mechanism by which hyperparathyroidism worsens and parathyroidectomy improves renal anaemia still remains unclear.

Meytes et al. [10] demonstrated that bovine parathyroid hormone (PTH) inhibits human and mouse erythroid burst-forming units directly. On the contrary, Delwiche and co-workers [11] reported that more purified bovine PTH had no inhibitory effect on human erythropoiesis. Therefore it is not known whether human PTH itself affects human erythropoiesis or not.

To clarify the direct effect of PTH on erythropoiesis, we examined colony assays of human haematopoietic progenitor growth.

Subjects and methods

Human N-terminal (1–34) PTH was purchased from Asahi Chemical Industry Co., Ltd (Tokyo, Japan) and human intact (1–84) PTH was kindly provided by Chugai Pharmacology Co., Ltd (Osaka, Japan). Although the biological activities of both PTHs have been reported [12,13], we measured adenylate cyclase (cAMP) activity in LLC-PK1 cells [14], a porcine renal tubular cell line with proximal tubular properties, to estimate the actual biological activity of the batches of both PTHs. The cells were obtained from Dainippon Seiyaku (Osaka, Japan), and were maintained in 15-cm² tissue culture flanks in medium 199 (Nissui Pharmaceutical Co., Ltd, Tokyo, Japan), supplemented with 5% fetal calf serum (FCS; Equitech-Bio, Inc, Ingram, TX), at 37 °C in a humidified incubator with 5% CO₂. LLC-PK1 cells were aliquoted into a 24-well plate (Sumitomo Bakelite Co., Ltd, Osaka, Japan) at a density of 1 × 10⁵ cells per well.

After 48 h, complete medium in all wells was replaced by serum-free medium containing 10⁻⁹, 10⁻⁸, 10⁻⁷, and 10⁻⁶ mol/l of N-terminal PTH or intact PTH for 10 min. Thereafter, these media were removed, and ice-cold 65% ethanol was added to each well. The supernatants were aspirated into tubes. The remaining precipitates were washed with ice-cold 6 5% ethanol, and the extracts were centrifuged at 2000 g for 15 min at 4 °C. The supernatants were transferred to fresh tubes. The combined extracts were dried in a vacuum oven. The dried extracts were dissolved in 100 μl of an assay buffer (0.05 M sodium acetate, pH 5.8 containing 0.02% bovine serum albumin (BSA, Sigma) and 0.01% preservative) prior to analysis. The concentration of cAMP was determined by enzyme immunoassay (cAMP EIA system,
Amersham, Buckinghamshire, UK). All tests were performed in triplicate.

Studies involving human subjects were carried out according to the principles outlined in the Declaration of Helsinki. Bone marrow samples were obtained from six healthy donors for bone marrow transplantation after obtaining informed consent, and diluted 10-fold with RPMI 1640-medium (Nissui Seiyaku Co., Tokyo, Japan) supplemented with 10% FCS. Mononuclear cells were separated by centrifugation on Ficoll–Hypaque (Pharmacia Fine Chemicals, Piscaway, NJ, USA) at 400 g for 30 min.

Human bone marrow blood colony-forming units-erythroid (CFU-e) and burst-forming units-erythroid (BFU-e) were carried out by the methylcellulose culture method described by Hirokawa et al. [16]. Briefly, 1 × 10^5 bone marrow cells in 1.0 ml of Iscove’s modified Dulbecco’s medium (IMDM; GIBCO, Grand Island, NY) supplemented with 30% FCS, 1% BSA, and 0.96% methylcellulose in the presence of 2 U/ml of human Epo (Kirin Brewery Co., Ltd, Tokyo, Japan) in 35-mm plastic Petri dishes (Lux, Miles Laboratories, Naperville, IL) for 7–14 days at 37°C in a high-humidity, 5% CO_2–95% air tissue incubator. Eight or more haemoglobinized cells were considered a CFU-e-derived colony. After about 14 days of incubation, BFU-e were evaluated when they consisted of 50 or more haemoglobinized cells or three or more clusters. Human granulocyte-macrophage colony-forming units (CFU-GMs) were assayed by a modification of the methylcellulose culture method described by Hirokawa et al. [16]. Briefly, 1 × 10^5 bone marrow cells in 1.0 ml IMDM containing with 0.96% methylcellulose, 20% FCS in the presence of 100 ng/ml of human granulocyte-macrophage colony stimulating factor (GM-CSF) (Kirin Brewery Co., Ltd, Tokyo, Japan) in 35-mm plastic Petri dishes (Lux, Miles Laboratories, Naperville, IL) for 7–10 days. The concentrations of both Epo and GM-CSF were determined by the preliminary study (data not shown). Aggregates containing more than 50 cells were scored as colonies.

Human N-terminal (1–34) PTH and human intact (1–84) PTH were added to cultures at the final concentrations of 300 ng/ml and 5000 pg/ml respectively. Since Lutton et al. [17] demonstrated that both exogenous human N-terminal (1–34) PTH and C-terminal [53–84] PTH concentrations ranging between 50 and 2000 pg/ml failed to inhibit mouse erythroid colony formation, we determined higher concentrations of both N-terminal PTH and intact PTH. With regard to amino-acid sequences, 11 amino acids differed from those of bovine intact PTH [18].

The effect of various concentrations of Epo on the inhibitory effect of both N-terminal PTH and intact PTH on erythroid colony formation was examined. Student’s t test was used to determine the significance of differences. A P < 0.05 was considered significant.

### Results

The biological activities of N-terminal (1–34) PTH and intact PTH were examined by measurement of LLC-PK1 cell adenylate cyclase assay as shown in Figure 1. These data demonstrated that both PTHs used in our study were actually active.

The effect of PTH on human bone marrow blood BFU-e, CFU-e, and CFU-GM are shown in Table 1.

In five of six healthy donors, neither human N-terminal PTH nor intact PTH showed inhibitory effects.
Table 2. Effect of increasing amounts of erythropoietin on the inhibitory effect of both human PTH (1–34) and intact PTH (1–84) on both human BFU-e and CFU-e

<table>
<thead>
<tr>
<th>Epo concentration (U/ml)</th>
<th>BFU-e</th>
<th></th>
<th>CFU-e</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PTH (1–34)</td>
<td>Intact PTH</td>
<td></td>
<td>PTH (1–34)</td>
</tr>
<tr>
<td></td>
<td>(300 ng/ml)</td>
<td>(5000 pg/ml)</td>
<td></td>
<td>(300 ng/ml)</td>
</tr>
<tr>
<td>0</td>
<td>100 ± 7.0 (%)</td>
<td>97 ± 5.7 (%)</td>
<td>121 ± 30.7 (%)</td>
<td>100 ± 18.4 (%)</td>
</tr>
<tr>
<td>1.0</td>
<td>100 ± 8.4 (%)</td>
<td>105 ± 6.1 (%)</td>
<td>115 ± 33.5 (%)</td>
<td>100 ± 12.6 (%)</td>
</tr>
<tr>
<td>2.0</td>
<td>100 ± 5.7 (%)</td>
<td>94 ± 12.1 (%)</td>
<td>108 ± 11.3 (%)</td>
<td>100 ± 5.8 (%)</td>
</tr>
</tbody>
</table>

Data shown are the mean ± SD (% of control mean) of three studies with each study made of triplicate plates.

Discussion

Secondary hyperparathyroidism is thought to play an important role in the genesis of anaemia of patients with CRF [6]. Indeed, many investigators have reported that uraemic patients with severe renal anaemia showed significant improvement of anaemia after partial parathyroidectomy [5,7–9]. Several researchers reported that PTH is a major uraemic toxin responsible for erythropoietic inhibition [10,19], and showed a direct inhibitory effect of PTH on erythropoiesis.

However, Delwiche et al. [11] could not reproduce the finding that purified preparations of bovine PTH inhibited either mouse or human erythropoiesis. Lutton et al. [17] estimated the effect of sera from uraemic patients on mouse CFU-e. They observed that 17 of 20 specimens inhibited CFU-e, and that high concentrations of human N-terminal (1–34) and C-terminal (53–84) PTH did not result in any inhibitory effect on CFU-e. Therefore they suggested that there may be circulating inhibitors of erythropoiesis, not PTH, in sera from uraemic patients. In the present study, we could not demonstrate that human N-terminal (1–34) PTH and intact PTH inhibited human erythropoiesis, except for an inhibitory effect of intact PTH on CFU-e growth in donor number 4, which may have been caused by a technical error. Therefore our findings support the results reported by Delwiche et al. [11] and Lutton et al. [17].

Other possible mechanisms of the improvement of renal anaemia after parathyroidectomy include an increase of haematopoiesis following an improvement of osteitis fibrosa and a reduction in bone marrow fibrosis [20,21], and an increased Epo production [21,22]. A good correlation between the presence of anaemia and the extent of marrow fibrosis was reported [8,9]. They suggested that the regression of marrow fibrosis after parathyroidectomy leads to an improvement of erythropoiesis. Rao et al. [21] demonstrated a correlation between resistance to erythropoietin and increased PTH with bone marrow fibrosis. Ureña et al. [20] and Washio et al. [22] reported that parathyroidectomy leads to a striking increase in serum Epo in patients with CRF.

In conclusion, since human PTH does not directly inhibit human erythropoiesis, other mechanisms including bone marrow fibrosis, or decreased Epo production may be responsible for resistance to erythropoietin in some uraemic patients.

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